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Scribbler is a dominant second site modifier of the *Drosophila Merlin* tumor suppressor gene. This year we have described a signal transduction circuit among *scribbler*, *Merlin* and *Cyclin E*. Using genetic epistasis, we show that *Merlin* functions upstream of both *scribbler* and *Cyclin E* – demonstrating that Merlin is a dominant second site repressor of loss of function phenotypes for Cyclin E. We also show that the two scribbler protein isoforms are not functionally equivalent; the smaller SbbA expression promotes ectopic proliferation via ectopic transcription of Cyclin E, while the larger SbbB represses proliferation by reducing the amount of Cyclin E expression. Using these data we have constructed an intriguing pathway for scribbler/Merlin regulation of proliferation. We propose Merlin functions by regulating the intracellular levels of the two scribbler isoforms. As we propose, in cells that are undifferentiated and actively proliferating, the expression of the smaller, proliferagenic SbbA isoform predominates, while in cells that have differentiated, Merlin may down regulate the expression of SbbA and promote the expression of SbbB. Currently we do not know whether Merlin may be regulating alternative splicing or the stability/instability of the mRNA of scribbler isoforms.

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Table of Contents

Cover.....

SF 298.....1

Table of Contents.....2

Introduction.....3

Body.....4

Key Research Accomplishments.....11

Reportable Outcomes.....12

Conclusions.....13

References.....14

Appendices.....16

Dennis LaJeunesse, Ph.D.
Department of Biology, UNCG
Genetic and Molecular characterization of *Drosophila brakeless*

Introduction

This is the annual report **Award (DAMD17-01-1-0718) entitled: "Genetic and molecular characterization of *Drosophila brakeless* (*scribbler*): a novel modifier of *Merlin* Phenotypes."**

As part of Specific Aim 1 of our Statement of Work, we have concentrated on characterizing the signal transduction pathway defined by *Merlin*, *scribbler* and *Cyclin E*. We believe this to be a major pathway involved in Neurofibromatosis type II tumor growth. We have shown that the two *scribbler* isoforms (*sbbA and sbbB*) are not functionally equivalent as previously suggested (Yang et al, 2000; Senti et al, 2000). SbbA appears to promote cell proliferation transcription of *Cyclin E*, while ectopic SbbB appears to repress proliferation. However, we do not know whether SbbB is repressing *Cyclin E* transcription or SbbA activating *Cyclin E* transcription. From genetic epistasis, we know that *Merlin* functions upstream of *scribbler* and *Cyclin E*. We are currently in the process of determining whether *Merlin* regulates the stability of *scribbler* protein isoforms or the alternative splicing of scribbler to generate altered ratios of scribbler isoforms. From our model we predict that cells undergoing rapid proliferation possess larger amounts of the small *scribble* SbbA isoform than cells that have undergone differentiation and that Merlin regulates the levels of *scribbler* isoforms. To test this hypothesis we have constructed RNA probes specific to each *scribbler* isoform and are currently using them to determine whether the expression of each isoform is altered in a *Merlin* mutant background.

We have identified a human homologue to *scribbler* (*HSbb*) which shares a similar genomic organization and appears to be alternatively spliced like the *Drosophila* gene. Northern analysis of several different cancer cell lines demonstrates the presence of two major isoforms in a pattern reminiscent to what is observed in *Drosophila*. We have generated FLAG and HA epitope-tagged versions of the HSbb isoforms in a human CMV expression vector (Invitrogen) which are for experiments that are designed to determine whether human cells have the same requirements for the *HSbb* as flies do for *scribbler* regarding the regulation of proliferation. We are also planning to generate *HSbb* transgenic *Drosophila* to test whether the fly and human proteins are functional homologues. In addition to further characterize the cellular function of *scribbler* we have generated three antibodies to *scribbler* proteins: one specific to the larger SbbB isoform, one antibody to the highly conserved Region A of the protein, and an antibody to the human scribbler conserved region.

As part Specific Aim I, we have attempted to characterize Merlin's subcellular vesicular bodies using enhanced yellow fluorescent protein constructs that have been targeted to various intracellular membrane bound compartment: the endoplasmic reticulum, the Golgi and the mitochondrion. Although none of this effort resulted in the elucidation of the exact nature of Merlin bodies, this work was published in **Biotechiques** in May, 2004.

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Genetic and Molecular characterization of *Drosophila brakeless*

Body:

In past reports we described a genetic interaction between *Merlin* and *Cyclin E*. In these genetic experiments, we show that *Cyclin E* is a dominant second site modifier of Merlin phenotypes expressed by a dominant negative allele of *Merlin* (Merlin $^{\Delta Blue\ Box}$; LaJeunesse et al, 1998) or loss of function allele *Merlin* (*Mer*^{3}; LaJeunesse et al, 1998; McCartney et al, 2000). In these experiments *Merlin* wing phenotypes were altered in a heterozygous *Cyclin E* mutant background using two different mutant Cyclin E alleles, *Cyclin E*^{10007} and *Cyclin E*^{05206} (data not shown). Dominant genetic interactions often predict an underlying interaction at a cellular and/or molecular level. Furthermore, we wished to examine whether mutations in *Merlin* reciprocally modified *Cyclin E* phenotypes. Since *Cyclin E* is an essential gene and flies homozygous for a null *Cyclin E* mutation die as embryos, we we unable to use null *Cyclin E* alleles to determine whether Merlin is a dominant modifier of *Cyclin E*. The *Cyclin E* mutant allele, *Cyclin E*^{jp1} (*CycE*^{jp1}) is a viable hypomorphic allele of Cyclin E that expresses a small rough eye phenotype as a result of a reduction of a pigment cells (Secombe et al, 1998). The molecular lesion of *CycE*^{jp1} is an insertion of a transposable element that results in a reduced expression the gene (Duman-Scheel, et al, 2002). Mutations in a number of known cell cycle regulators have been shown to enhance *CycE*^{jp1} phenotypes in a dominant second site manner; that is flies homozygous for the *CycE*^{jp1} mutation and heterozygous for another mutation express a phenotype more severe that flies homozygous for *CycE*^{jp1} alone. Some of the Enhancers of *CycE*^{jp1} described in Secombe et al, 1998 include genes that encode proteins known to regulate the activity of *Cyclin E* through protein-protein interactions. These include genes such as *dacapo* (*Drosophila* p21), retinoblastoma, and *cdc2c* (Secombe et al, 1998). The interpretation for these genetic interactions is that a quantitative reduction of proteins essential for *Cyclin E* function -- as a result of heterozygosity for the mutation in such genes -- results in a qualitative worsening of *CycE*^{jp1} phenotypes. However, genetic interactions also identify genes that are involved in *Cyclin E* function in circuitous manner. For instance genetic modifiers of *CycE*^{jp1} also included genes encoding products involved in the G2/M transition (Secombe et al., 1998). In these cases, the enhancement of the *CycE*^{jp1} phenotype was interpreted as a result of less cells entering mitosis in these genetic combinations.

We have discovered that *Merlin* is a dominant second site suppressor of *CycE*^{jp1} phenotypes (figure 1). Flies homozygous for the *CycE*^{jp1} mutation and heterozygous for a mutation in *Merlin* express a phenotype <u>less</u> severe (Figure 1 E and F) than flies homozygous for *CycE*^{jp1} alone (figure 1 C and D). We have performed the experiment with two different alleles of *Merlin* – a null *Mer*^{4} allele and a hypomorphic *Mer*^{3} allele. In each case, the results were similar with the null *Merlin* allele expressing a slightly greater suppressor of the *CycE*^{jp1} eye phenotype. We also found that the *CycE*^{jp1} eye phenotype is temperature sensitive. At 29°C the CycE^{jp1} eye phenotype is more severe and we observed a similar suppression of the more severe *CycE*^{jp1} phenotype (figure 1 compare C and D). Our interpretation of the genetic interaction between *Merlin* and *Cyclin E* suggests that *Merlin* is involved with the <u>negative</u> regulation of *Cyclin E* expression or function. This negative regulation could be either through regulation of *Cyclin E* transcription or degradation of *Cyclin E* protein as both

mechanisms play a role in the transition through the cell cycle (Richardson et al, 1995). Moreover, *Merlin* might also regulate the function of expression of other *Cyclin E* regulators such as *dacapo* and cdk2. However, in light of other evidence we have found, we believe that *Merlin* regulates *Cyclin E* transcription through *scribbler*.

Scribbler is also dominantly modified by *Cyclin E* mutations (data not shown). In these cases, loss of function alleles of *scribbler* and *Cyclin E* exhibit a slight dominant second site modification. These results further suggest that *Merlin* and *scribbler* function together with *Cyclin E* to regulate growth. However we plan to determine whether *scribbler* also acts as a dominant modifier of $CycE^{jp1}$ phenotypes as *Merlin* does and we have constructed the four different recombinant chromosomes that bear the $CycE^{Jp1}$ mutant allele and one of four different *scribbler* alleles. We choose to use to use the null *scribbler* allele, sbb^{4}, the hypomorphic *scribbler* allele sbb^{032027}, and two *Enhancer of Merlin* alleles of scribbler, sbb^{256} and sbb^{324}.

Figure 1. Merlin is a Dominant Suppressor of hypomorphic _Cyclin E_ phenotypes

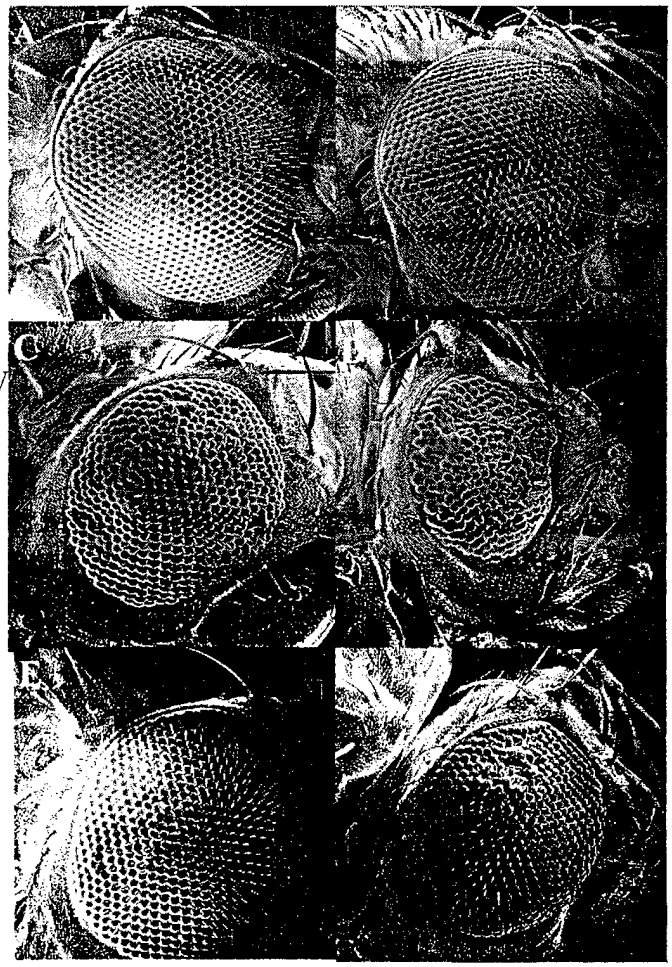

Figure 1. Mutations in *Merlin* dominantly suppress the recessive rough eye phenotype expressed by a hypomorphic *Cyclin E* mutation. A) Wild type adult eye at ~20°C, B) Wild type adult eye at 29°C, C) $CycE^{jp1}/Cyc\ E^{jp1}$ adult eye at ~20°C, D) $CycE^{jp1}/CycE^{jp1}$ adult eye at 29°C *E.* $mer^{4}/+$; $Cyc\ E^{jp1}/CycE^{jp1}$ adult eye at ~20°C; F) $mer^{4}/+$; $CycE^{jp1}/CycE^{jp1}$ adult eye at 29°C. The adult *Drosophila* eye contains 400 ommatidia arranged in a highly organized array . Homozygosity for $CycE^{Jp1}$ results in a smaller, more disorganized arrangement of ommatidia due to reduced number of pigment cells (Secombe et al, 1998). The $CycE^{Jp1}$ eye phenotype is temperature sensitive, compare C and D. In a CycEjp1 mutant background, heterozygosity for a null Merlin allele (Mer^{4}) suppresses this phenotype, compare E to C and F to D. At 20°C this phenotype is suppressed almost to wild type size.

This experiment also helps us with an objective in Specific Aim 1 in which we planned to determine whether there were qualitative differences between *scribbler* mutations regarding

proliferation and interactions with *Merlin*. Null alleles of *scribbler* that eliminate both isoforms of *scribbler* do not significantly modify Merlin phenotypes (LaJeunesse et al, 2000), therefore we predict that only the 'Enhancer of *Merlin*' *scribbler* alleles (which have molecular lesions that only affect the *SbbB* isoform) will behave like loss of function *Merlin* mutations.

Scribbler was simultaneously identified by five independent groups: two working on axon guidance in the developing retina of the *Drosophila* eye (Senti et al, 2000; roa et al., 2000), one in a screen for mutations that affect feeding behavior (Yang et al, 2000), another working on genes that regulate *decapentapelgic* signaling in the developing wing disc (Funakoshi et al., 2001), and as a modifier of *Merlin* phenotypes (LaJeunesse et al, 2001). In an initial characterization of *scribbler*, both isoforms were shown to rescue the lethality and axon guidance defects associated with loss of *scribbler* function (Senti et al., 2000). However, we have demonstrated that although there appears to be redundant function shared between small and large scribbler isoforms, expression of the smaller 929 amino acid long *SbbA* isoform results in an increase of cell proliferation. When expressed in the posterior compartment of the wing (figure 2A), ectopic expression

Figure 2: Ectopic expression of SbbA and

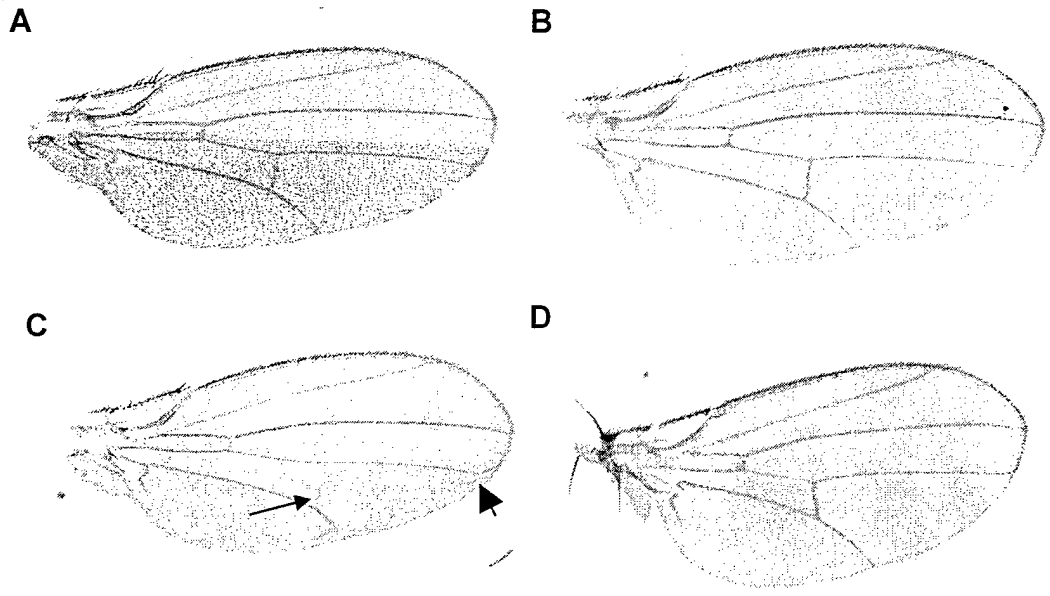

Figure 2: Two *scribbler* isoform work in opposite fashions regarding proliferation of wing cells. All wings are from three day old adult female flies. A) wild type adult wing with the posterior compartment shaded. B) A adult wing expressing *UAS::SbbA* (the small scribbler isoform) under the *engrailed-G*al4 in posterior compartment of wing. Notice the increase in the area when compared with wild type wing. C) Wing expressing *UAS::SbbB* (the large scribbler isoform) under the engrailedGal4 in posterior compartment of wing. Notice the decrease in the area when compared with the wild type wing and loss of the posterior cross vein (small arrow) and ectopic material along wing margin. D) A wing co-expressing *UAS::SbbA* and *UAS::SbbB*. Notice how wing's size is closer to wild type and how defects in venation seen with expression of *SbbB* are not present.

of *sbbA* results in an increase in surface area without an increase in size of the cells (figure 2B). We observe the same phenotype when we express *sbbA* using different expression systems. We also observe an increase in the amount of incorporation of BrdU in the posterior compartment in the developing wing imaginal disc (data not shown). In contrast, expression of the larger 2028 amino acid *SbbB* isoform results in a marked decrease in size of the posterior compartment (figure 2C). The decrease in size does not appear to be due to ectopic apoptosis, as co-expression of a bacculovirus P35 protein which inhibits apoptosis does not ameliorate this phenotype. In addition, expression of the *SbbB* isoform also results in alteration of venation with the absence of the posterior cross vein and formation of ectopic deltas at the termini of the longitudinal veins (figure 2C, arrows). Interestingly, co-expression of the SbbA and SbbB isoforms results in the reversion of the overgrowth phenotype observed with ectopic expression of SbbA and the venation defects associated with ectopic SbbB expression. This result suggests to us that the relative levels of large and small *scribbler* isoforms are regulated during development to ensure proper growth and differentiation of wings. This result also suggests that a mechanism is in place to regulate the levels of these isoforms.

Several labs have shown that *scribbler* functions as a negative regulator of transcription. Funakoshi et al, 2001 showed that *scribbler* (in this paper known as *master of thick veins*; *mtv*) operated downstream of *hedgehog* signaling and decreased the transcription of *thick veins* in a domain along the anterior/posterior boundary when *dpp* is expressed and secreted. They hypothesize that this transcriptional repression in effect increased *dpp* signaling throughout the wing by permitting the *dpp* ligand to freely move across the wing epithelium (Funakoshi et al, 2001). It has been shown that the axon guidance phenotypes observed in *scribbler* loss of function developing eyes (Kaminker et al, 2002) is due to the de-repression of a gene called *Runt* in a set of key pioneering photoreceptor axons. *Runt* encodes a transcription factor that is normally repressed in the R2 and R5 photoreceptors. In *scribbler* (known in these papers as *brakeless*; *bks*) mutants RUNT is ectopically expressed in these two photoreceptors resulting in a improper trajectory of all photoreceptor axons projecting in to target sites within the optical regions of the Drosophila central nervous system (Kaminker et al, 2002).

To examine whether *scribbler* transitionally regulates *Cyclin E,* we examined the expression levels of a *Cyclin E* LacZ reporter (*16.4 CycE LacZ*) construct in wing imaginal discs that over-expressed either the large or the small scribbler isoform. *16.4 CycE LacZ* contains 16.4 kb of DNA upstream of the *Cyclin E* coding region and has been shown to faithfully expression the reporter enzyme beta-galactosidase (*lacZ*) in a pattern reminiscent of normal *Cyclin E* transcription (Jones et al, 2000). In flies expressing *SbbA* in an engrailed expression pattern, we observed an increase of *16.4CycE LacZ* expression (figure 3, lane 6). Moreover, this increase in transcription correlates with an observed increase in *Cyclin E* protein levels (figure 4, lanes 3 and 5). Although we did not observe a measurable decrease in the amount of transcription from the *16.4CycE LacZ* reporter construct when we over expressed *SbbB* (figure 3, lane 5), we did see a decrease in *Cyclin E* protein levels in discs expressing *SbbB* (figure 4, lanes 4 and 6) suggesting that ectopic expression of *SbbB* might play additional

although non-direct roles in *Cyclin E* regulation. These experiments establish *scribbler*'s requirement for proper *Cyclin E* expression.

Figure 3: Ectopic expression of scribbler isoforms alters *16.4 Cyclin E lacZ* reporter gene expression

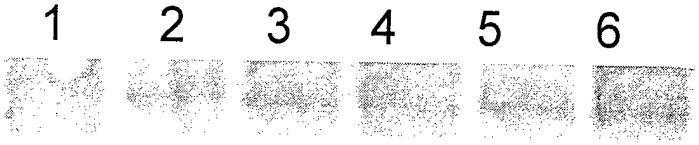

Figure 3. Cyclin E reporter genes levels are altered by ectopic expression of scribbler isoforms. **Lane 1**: w^{1118} extract (no reporter gene present) ; **Lane 2**: heterozygous *CycE LacZ* extract; **Lane 3**, homozygous *CycE LacZ* extract; **Lane 4**, homozygous *CycE LacZ* extract; **Lane 5**, *enagrailed-Gal4 UAS::SbbB; CycE LacZ/+* extract; **Lane 6**, *engrailed-Gal4 UAS::SbbA*; Cyc E LacZ/+ extract. Equivalent quantity of protein (40ug) were loaded per lane, as verified by Bradford assay and independent re-probing with anti-Tubulin antibody. Ectopic expression of UAS::SbbA under *engrailed*-Gal4 (lane 5) expression system results in greater 16.4 Cyclin E-lacZ expression levels when compared to wild type (lane 2, 3 or 4).

Figure 4: Ectopic expression of scribbler isoforms alters Cyclin E expression

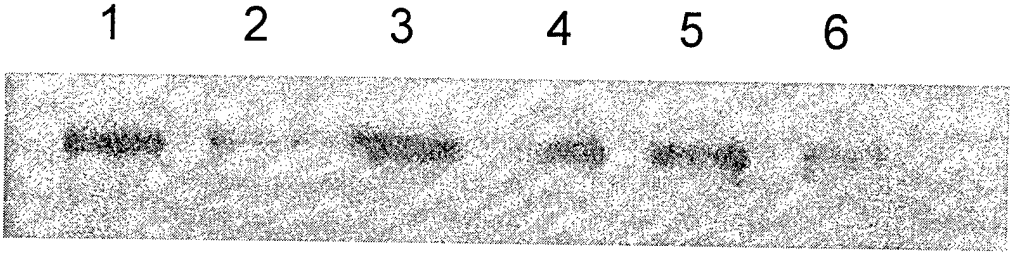

Figure 3. Cyclin E protein levels are altered by ectopic expression of scribbler isoforms. Lane 1: wild type wing disc extract ; lane 2: $CycE^{jp1}$ homozygous wing disc extract; lane 3, *apterous-Gal4 UAS::SbbA* wing disc extract; lane 4, *apterous-Gal4 UAS::SbbB* wing disc extract lane 5, *enagrailed-Gal4 UAS::SbbA*; lane 6, *engrailed-Gal4 UAS::SbbB* wing disc extract. Each lane has the equivalent quantity of protein(30 imaginal discs were dissected and used to make the extract) This was verified by Bradford assay and independent re-probing with anti-Tubulin antibody. Ectopic expression of UAS::SbbA under either *apterous* Gal4 (lane 3) and *engrailed*-Gal4 (lane 5) expression system results in greater Cyclin E levels when compared to wild type (lane 1). Conversely, ectopic expression of UAS::SbbB under the same drivers (lanes 4 and 6) results in less Cyclin E protein levels, compare to wild type (lane 1) and to CycEjp1 homozygous wing discs (lane 2).

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Genetic and Molecular characterization of *Drosophila brakeless*

We are currently comparing the promoter regions of *Cyclin E*, *thick veins* and *Runt* to determine whether there are sites that are common between these elements. A well conserved tryptophan-rich basic region (Region A) in the *scribbler* proteins suggests that *scribbler* proteins might have site specific DNA binding capabilities. Although an overlapping subset of genes might be negatively regulated by both *scribbler* isoforms it is possible that at certain loci such as *Cyclin E*, the SbbB isoform might function to repress transcription while SbbA does not.

We have developed a model of *Merlin* function regarding *scribbler* and the regulation of *Cyclin E* expression. In our model we hypothesize that in response to growth factor cessation *Merlin* switches the expression of scribbler isoform from the proliferagenic SbbA to a non-proliferagenic SbbB. This switch does not necessarily involve a complete change in the presence or absence of an isoform but could simply result in a change in the ratio of one isoform versus the other. We are currently testing this hypothesis with two experiments. The first experiment involves performing *in situ* hybridizations with probes specific to the message encoding the large *scribbler* isoform, to the message encoding the small scribbler isoform and a probe common to both *scribbler* isoforms in a *Merlin* mutant background to examine whether there is an alteration of scribbler isoform due to the loss of *Merlin* function. We would expect to see an increase in the message encoding the smaller *scribbler* isoform in *Merlin* loss of function mutants. In addition to this we are also performing Western analysis comparing *scribbler* protein levels from extracts from wild type and *Merlin* mutant larvae. While either experiment would confirm our hypothesis that *Merlin* is regulating the level of scribbler isoforms, together they tell us whether the regulation is at the level of the message or at the protein level.

Although the mechanism of a putative Merlin-induced regulation of *scribbler* isoforms has not been identified we imagine that there are three possible mechanisms: the regulation of alternative splicing, the regulation of differential mRNA stability, and differential protein degradation. Recently the alternative splicing of CD44 has been demonstrated to be downstream of extracellular signal-regulated kinase through the activation of a RNA binding protein called Sam68 (Matter et al, 2002). Given that Merlin has been shown to down regulate both *Rac/cdc42* function in mouse and human tissue culture cells (Shaw et al, 2001; Xiao, et al, 2002), it seems likely that one output of these pathways could be the regulation of Sam68 or a Sam68 like protein. Moreover, homologues exist in flies to the family of RNA binding protein to which Sam68 is a member (Fruscio et al, 1998) Included in this family is the gene *held out wing* (*how/who)*, which has been identified in a number of screens (Fyrberg et al, 1997; Baehrecke, 1997). One possible model of *Merlin* function regulates *scribbler* expression via growth-factor/Rac signally responsive protein like how. Interestingly, the *how* phenotype is photocopied in both *Merlin* and *scribbler* mutant flies. Currently, we are testing whether *how/who* has a genetic interaction with *Merlin* or *scribbler* mutant flies.

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Biotechniques paper summary and intentions

As a separate objective in Specific Aim 1, we wanted to determine the nature of the intracellular Merlin protein containing bodies that are found in *scribbler* mutant epithelial cells. To do this we constructed three different Green Fluorescent Protein intracellular membrane markers and are examining their co-localization with Merlin within various tissues. In our studies we discovered that Merlin was not a component of the Golgi, endoplasmic reticulum or mitochondrial network. Nor were the aberrant Merlin localization due to experimental artifact. Moreover, we observed the aberrant localization of Merlin in different genetic backgrounds suggesting that aberrant Merlin localization may be the result of a more complicated phenomenon. We published our findings in **Biotechniques** May 2004 issue. A copy of the manuscript is attached in the appendix.

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Genetic and Molecular characterization of *Drosophila brakeless*

Key Research accomplishments:

- **Delineation of a *Merlin* mediated signal transduction pathway that provides a clear means for the regulation of proliferation via *Cyclin E* transcription.**
- **Generation of antibodies to scribbler proteins**
- **Initiation of the characterization of a human scribbler homologue**

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Reportable Outcomes:

- **Poster presentations at the 45th annual National *Drosophila* convention in Washington D.C: Poster #465C**

***Merlin* and *scribbler* isoforms regulate *Cyclin E* expression and cellular proliferation**
Dennis LaJeunesse, Stephanie Buckner, Rebecca Atwell, Amanda Brown, and Amanda Pirt. University of North Carolina Greensboro, 231 Eberhart Bldg., Greensboro, North Carolina 27455
Scribbler was identified as a dominant second site modifier of the *Drosophila Neurofibromatosis type II* tumor suppressor gene homologue, *Merlin*. Mutations in *Drosophila Merlin* result in defects in the regulation of proliferation as well as defects in differentiation. We have continued the work on the circuit between *scribbler*, *Merlin* and *Cyclin E*. Using epistasis, we show that *Merlin* functions upstream of *scribbler*. We also demonstrate that the two scribbler isoforms (SbbA and SbbB) are not equivalent in function. Ectopic SbbA expression appears to promote cell proliferation, while ectopic SbbB appears to repress proliferation. Moreover, both *scribbler* and *Merlin* genetically interact with both loss and gain of function mutations of *Cyclin E* suggesting a common mechanism in the regulation of proliferation. In addition to this, we demonstrate that Sbb regulates Cyclin E transcription. With these data we have constructed an intriguing hypothetical pathway for sbb/Merlin regulation of proliferation: *Merlin* may regulate the alternative splicing *of scribbler*, which in turn affects Cyclin E transcription. We have also identified a human homologue to scribbler (HSbb) which shares a similar genomic organization to the *Drosophila scribbler* gene. We will present preliminary data on whether HSbb is alternatively spliced like its Drosophila homologue and plays a similar role in the regulation of proliferation.

- Manuscript in BioTechniques entitled: "Three new *Drosophila* markers of Intracellular Membranes and Fixation Artifact."

- Manuscript in preparation entitled: "*Merlin* and *scribbler* regulate *Cyclin E* expression and cellular proliferation."

- Generation of three new antisera to *scribbler*.

- Generation of five RNA probes specific to *SbbA* and *SbbB* isoforms.

- The generation of FLAG and HA tagged HSbb transgenes for examination in human tissue culture cells.

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Genetic and Molecular characterization of *Drosophila brakeless*

Conclusions:

- Establishment that alternatively splice *sbb* isoforms have unique roles in differentiation and proliferation
- Demonstration that *Merlin* is a dominant suppressor of *Cyclin E* phenotypes.
- Identification of a signal transduction pathway by which *Merlin* and *scribbler* take part to regulate *Cyclin E* expression and cellular proliferation.
- Aberrant *Merlin* protein localization observed in *scribbler* mutant epithelium is not a component of the Golgi, endoplasmic reticulum or mitochondrial network.

References

Baehrecke, E.H. (1997) *who* encodes a KH RNA binding rpotien that functions in muscle development. Development 1223-1332.

Brand, A. H. and Perrimon, N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118, 401-415.

Di Fruscio M, T. Chen, S. Bonyadi, P. Lasko, S. Richard (1998) The Identification of Two Drosophila K Homology Domain Proteins, The Journal of Biological Chemistry. 46, 30122-30130.

Duman-Scheel, M, L. Weng, S. Xin, W. Du (2002) Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. Nature 427, 299-304

Funakoshi Y., M.Minami and T. Tabata (2001) *Mtv* shapes the activity gradient of the Dpp morphogen through regulation of *thickveins*. Development 128, 67-74

Fyrberg, C., J. Becker, P. BArthnaier, J. Mahaffey, E. Fryberg, (1997) A Drosophila muscle-specific gene related to the mouse quaking locus. Gene 197, 315-323.

Kaminker J., J. Canon, I. Saliker, U. Banerjee (2002) Control of photoreceptor axon target choice by transcriptional repression of Runt. Nature Neuroscience, 5: 746-750.

Kikuno R., Nagase T., Waki M. (2002). HUGE: a database for human large proteins identified in the Kazusa cDNA sequencing project. *Nucleic Acids Res.* **30**, 166-168

Kissil J. L., K. C. Johnson, M. S. Eckman, T. Jacks (2002) Merlin Phosphorylation by p21-activated Kinase 2 and Effects of Phosphorylation on Merlin Localization. 27, 10394–10399

LaJeunesse, D. R., McCartney, B. M. and Fehon, R. G. (1998). Structural analysis of Drosophila Merlin reveals functional domains important for growth control and subcellular localization. *J. Cell Biol.* 141, 1589-1599.

McCartney BM, Fehon RG. (1996) Distinct cellular and subcellular patterns of expression imply distinct functions for the Drosophila homologues of moesin and the neurofibromatosis 2 tumor suppressor, Merlin. Cell Biol. 133(4):843-52.

Matter, N, Heerlich, Konig, H (2202) Signal dependent regulation of splicing via phosphoryaltion of Sam68. Nature 420, 691-695.

Richardson H., L. V. O'Keefe, T. Marty, and R. Saint (1995) Ectopic Cyclin E expression induces premature entry into S phase and disrupts pattern formation in the *Drosophila* eye imaginal disc. Development 121, 3371-3379.

Rao, Y., Pang, P., Ruan, W., Gunning, D. and Zipursky, S. L. (2000). *brakeless* is required for photoreceptor growth-cone targeting in *Drosophila. Proc. Natl. Acad. Sci. USA* 97, 5966-5971.

Scanlan, M.J., J.D. Gordan, B. Williamson, E. Stockert, N.H, Bander, V. Jongeneel, A.O. Gure, D. Jager, E. Jager, A. Knuth, Y.-T. Chen, L.J. Old, (1999) Antigens recognized by autologous antibody in patients with renal-cell carcinoma Int. J. Cancer 83 (4), 456-464.

Secombe J., J. Pispa, R. Saint and H. Richardson (1998) Analysis of a *Drosophila cyclin E* Hypomorphic Mutation Suggests a Novel Role for Cyclin E in Cell Proliferation Control During Eye Imaginal Disc Development. Genetics 149: 1867–1882.

Senti K., K. Keleman, F. Eisenhaber, B.J.Dickson (2000) brakeless is required for lamina targeting of R1-R6 axons in the Drosophila visual system. Development. 127(11):2291-301.

Shaw R.J., Paez J.G., Curto M., Yaktine A., Pruitt W.M., Saotome I. O'Bryan J.P., Gupta V., Ratner N., Der C.J., Jacks T., McClatchey A.I. (2001) The Nf2 tumor suppressor, merlin, functions in Rac-dependent signaling. Dev Cell. 1. 63-72.

Yang P., S. A. Shaver, A. J. Hilliker, and M. B. Sokolowski (2000) Abnormal Turning Behavior in Drosophila Larvae: Identification and Molecular Analysis of *scribbler* (*sbb*) Genetics 155: 1161–1174.

Xiao, G., A. Beeser, J. Chernoff, J.R. Testa (2001) p21-activated Kinase Links Rac/Cdc42 Signaling to *Merlin*. Journal of Biological Chemistry, 883-886

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Genetic and Molecular characterization of *Drosophila brakeless*

Appendices:

Biotechnique manuscript:

Three new *Drosophila* markers of intracellular membranes

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The need for cellular markers that permit a quick and accurate evaluation of a protein's subcellular localization has increased with the surge of new data generated by the Drosophila *genome project. In this report, we present three ubiquitously expressed* Drosophila *transgenes that expressed a green fluorescent protein variant (enhanced yellow fluorescent protein) that has been targeted to different intracellular membrane targets: the Golgi apparatus, mitochondria, and endoplasmic reticulum. These markers serve as an internal standard for characterizing a protein's subcellular localization or as a means of tracking the dynamics of intracellular organelles during normal or abnormal cellular or developmental processes. We have also examined fixation artifacts using these constructs to illustrate the effects that fixation and permeabilization have on intracellular membrane organization.*

INTRODUCTION

With the completion of the *Drosophila* genome project, there has been a trend in *Drosophila* research toward assessing gene function at the cellular level. As the field of functional genomics races toward a comprehensive understanding of gene function, a need arises for intracellular standards that permit a quick and accurate evaluation of a novel protein's localization within the cell. Fusion proteins to green fluorescent protein (GFP) and its variants have been used to detect a target protein's subcellular localization in both living and fixed cells (1–3). Here we describe three *Drosophila* transgenes that ubiquitously express a GFP variant, enhanced yellow fluorescent protein (EYFP), which has been targeted to different intracellular membrane bound compartments: the Golgi apparatus, the endoplasmic reticulum, and the mitochondria. While extensive work has been performed on fixation and fixation artifacts in electron microscopy, there is little or no literature dealing with such topics in fluorescent light microscopy, especially regarding fixation of whole tissues in a multicellular model organism. Because EYFP can be examined in living and fixed preparations, we took the opportunity to examine the effects of fixation and permeabilization on the expression and localization of the targeted EYFP moiety.

MATERIALS AND METHODS

Microscopy

All images (both live and fixed) were captured using a fluorescein isothiocyanate (FITC) filter (Chroma Technology, Rockingham, VT, USA) and a 60× UPlanFl oil-emersion objective [numerical aperture (N.A.) 1.25] with oil on an Olympus BX51 compound fluorescent microscope (Olympus America, Melville, NY, USA) equipped with a Cool Snap *fx* charge-coupled device (CCD) digital camera (Photometrics, Tucson, AZ, USA). The camera and microscope were controlled using Image Pro® Software. Captured images were imported into Microsoft® PowerPoint® for presentation.

Sample Preparation

Tissue from wandering third instar larvae was dissected in Shields and Sang M3 Insect tissue culture media (Sigma, St. Louis, MO, USA). Living tissue was mounted on a glass microscope slide in a wet mount of 20 µL cell culture media under a 22-mm coverslip prepared with clay feet to prevent damage to the tissue. Fixed tissue was mounted using the ProLong® Antifade Kit (Molecular Probes, Eugene, OR, USA) and examined within 24 h of preparation because formaldehyde-based fixes tend to degrade. We observed little difference in tissue quality within 24 h of fixation.

Cloning and P-Element Germline Transformation

The membrane-targeted moieties were PCR-amplified from mammalian expression vectors pEYFP-Mito, pEYFP-ER, and pEYFP-Golgi (for the mitochondria, endoplasmic reticulum, and Golgi apparatus, respectively; BD Biosciences Clontech, Palo Alto, CA, USA) and cloned into a *Drosophila spaghetti squash* (*sqh*) CASPER 4 P-element transformation vector (gift of D. Kiehart) (Figure 1). EYFP is a brighter, red-shifted variant of the original jellyfish GFP. The *sqh* promoter expresses ubiquitously, permitting examination in a wide variety of tissue types without induction. P-element-mediated germline transformation was performed as previously described (3).

Initial Characterization of EYFP Constructs in Insect Cells

Because the EYFP constructs were designed for expression in mammalian tissue culture cells, we first determined whether the same organelles were labeled in *Drosophila* cells. Although the localization of each construct appeared to be similar in both human HeLa tissue culture cells and *Drosophila* Schneider line 2 tissue culture cells, we confirmed the localization in *Drosophila* tissue culture cells by double labeling the transfected cells with a fluorescent dye that specifically labeled each organelle, using MitoTracker Orange CMTMRos for the mitochondria, $NBDC_6$-ceramide for the Golgi apparatus, and ER-Tracker™ Blue-White DPX for the endoplasmic reticulum (all from Molecular Probes). In each case, we found that the signals overlapped perfectly, suggesting that our constructs labeled the intended structures in *Drosophila* cells (data not shown). Three independent insertions of each construct were characterized,

and each displayed subcellular patterns of localization to specific compartments that we interpreted as analogous to the structures we identified in the tissue culture.

Buffer Preparation

Buffer compositions are as follows: phosphate-buffered saline (PBS); phosphate lysine periodate (PLP), 0.037 M sodium phosphate, 0.075 M lysine, 0.01 M sodium periodate, pH 7.2; and PIPES/EGTA/magnesium (PEM), 0.1 M PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.9. An 8% paraformaldehyde stock solution was prepared by dissolving 80 g of paraformaldehyde in 80 mL of warmed water and 10 µL of 10 N NaOH and heated to 55°C for 2–3 h, distilled water was added to 100 mL, and the solution was vacuum-filtered to remove the flocculent nonsoluble fraction. To make fixatives, an appropriate amount of this paraformaldehyde stock solution was diluted in the desired concentrated buffer solution. In 1× PEM and 1× PBS buffers, a concentration of either 2% or 4% of paraformaldehyde was used; PLP fixative was made with 2% paraformaldehyde. Samples were fixed for 20 min at either 4°C or 21°C. To test for the effects of permeabilization, a 30-min wash of 1× PBS, 1% bovine serum albumin (BSA), and 0.1% Triton® X-100 (Sigma), called PBT, was performed on fixed samples.

RESULTS

We examined the expression of each construct in several different tissues, including muscles, neuronal cells, and epithelial cells (imaginal discs, guts, and salivary glands). In general, *Drosophila* cells are small; thus, in this report, we used the giant epithelial cells of the third instar larval salivary gland to demonstrate our results. These cells are large (approximately 100 µm in diameter) and permit easy observation of the subcellular localization of each of the organellar/intracellular membrane-targeted EYFP constructs. Considering that all immunological protocols involve a fixation step and that EYFP can be visualized in both living and fixed tissues, we examined the effects of fixation on the organization and distribution of intracellular membrane structure. We tested two concentrations of paraformaldehyde-based fixative (2% or 4%) in three buffer solutions commonly used in *Drosophila* research: PBS, PEM, and PLP, which has been reported to work well for membrane-associated epitopes (4,5). Not surprisingly, in all cases, we observed some degree of alteration of the subcellular EYFP patterning after fixation, regardless of the buffer used. We also examined the effects of fixation at two different temperatures [room temperature and on ice (4°C)] but saw no difference in results between these conditions (data not shown).

EYFP-Golgi showed a punctate distribution through the cytoplasm of a wide range of cells types, including salivary gland (Figure 2A), neuron, muscles, and intestinal cells (data not shown). While fixation does not alter the overall distribution of the EYFP-Golgi punctuate-labeled bodies, we observed subtle differences in the morphology of these structures after fixation, especially in the PLP buffer (compare Figure 2, A and D). In this buffer, we consistently observed finer distribution of Golgi throughout the cell. We do not know the basis for this observation. We also observed alteration in the EYFP-Golgi body organization as a function of paraformaldehyde concentration. Living EYFP-Golgi are composed of irregularly shaped, lobular structure (Figure 3A, inset). Fixation in 2% paraformaldehyde in PBS buffer resulted in a bloated, swollen appearance to these structures, perhaps as a consequence of incomplete fixation (Figure 3B, inset). Fixation in 4% paraformaldehyde fixative in a PBS buffer resulting in a rounded, smaller, more regularly shaped appearance (Figure 3C, inset).

EYFP-Mito was also distributed in punctate structures throughout the cytoplasm in salivary gland cells (Figure 2I). Furthermore, we noticed that EYFP-Mito also had a strong localization at cell cortex (Figure 2I, arrowhead) and an apical localization in the salivary gland epithelium basal to the adherens junction region, as determined by the localization of Merlin (data not shown; D.R. LaJeunesse, unpublished observations). The fixation of EYFP-Mito in all buffer conditions used resulted in loss of the cortical mitochondrial localization, while overall, the punctuate distribution of the mitochondria seemed relatively unperturbed (compare Figure 2I with J, K, and L).

In living salivary gland epithelial cells, EYFP-ER labeled a reticular net-

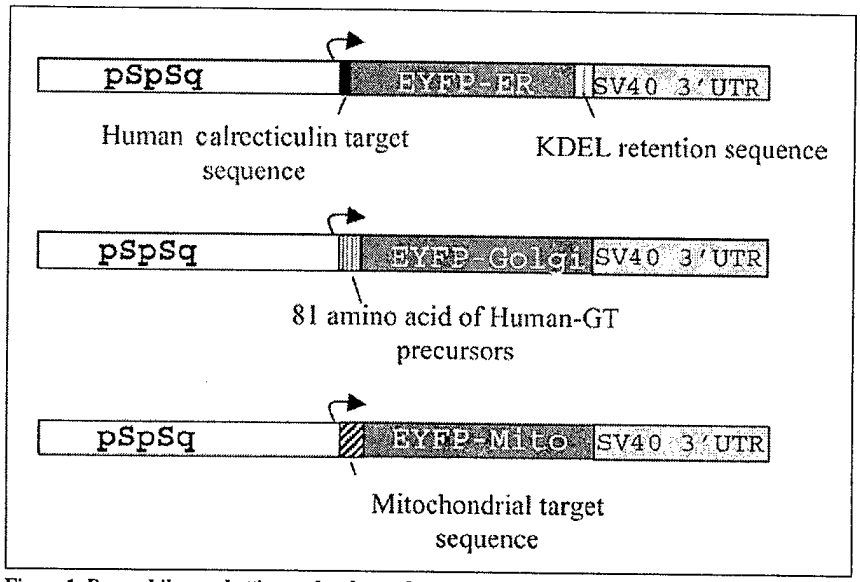

Figure 1. *Drosophila spaghetti squash* enhanced yellow fluorescent protein (EYFP) membrane-targeting constructs. Mammalian expression vectors pEYFP-ER, pEYFP-Golgi, and pEYFP-Mito were used to target sequence for the endoplasmic reticulum, Golgi apparatus, and the mitochondria, respectively. SV40, simian virus 40; UTR, untranslated region.

work found throughout the cytoplasm (Figure 2E). This reticular pattern was observed in different tissue types in varying degrees, ranging from completely filling the cytoplasm to a fine diaphanous network (data not shown). In this case, the pattern resembled that of a GFP fusion with an endoplasmic reticulum-specific protein disulfide isomerase (6). Interestingly, while the Golgi and mitochondria maintained some of the structural integrity that was observed in living tissue after fixation with a paraformaldehyde-based fixative, fixation of EYFP-ER resulted in complete loss of all observable structure. The result is an amorphous mass that occupies the cytoplasm and that is completely devoid of any discernable reticulum organization (compare Figure 2E with F, G, and H).

Since many immunological localization techniques contain a permeabilization step that usually involves a wash with a buffered solution containing a detergent, we wished to examine the effects on the organization and appearance of our labeled structures by treating the fixed tissue with a detergent wash. As our detergent, we used standard concentration 0.1% of a common reagent, Triton X-100. After fixation, we exposed the samples (either EYFP-Golgi or EYFP-Mito salivary glands) to a 30-min incubation/wash with PBT. This step is similar in both composition and duration to the blocking and incubations steps found in many common immunohistological protocols used in *Drosophila* research. As in the previous experiments, we fixed under several different buffered conditions and used either a 2% or 4% concentration of paraformaldehyde. These results are shown in Figure 3. As stated before, fixation with paraformaldehyde resulted in alterations of Golgi morphology (compare Figure 3A and inset with B and C). Furthermore, poorer preservation translated into a complete loss of label after incubation with buffer containing 0.1% Trition X-100 (compare Figure 3, B and D), while stronger fixation preserved the Golgi morphology better (compare Figure 3, C and E).

DISCUSSION

Subcellular localization is a powerful aid in determining gene function. Here we report the characterization of three new EYFP intracellular membrane markers for use in the *Drosophila* model system (*Sqh::EYFP-Golgi*, *Sqh::EYFP-ER*, and *Sqh::EYFP-Mito*) that label the appropriate intracellular organelles in living and fixed tissues and that permit the assessment of subcellular structures in living and fixed tissues and cells. These constructs will be of great use to the *Drosophila* research community, and stocks containing these constructs have been deposited at the Bloomington *Drosophila* Stock Center at Indiana University (Bloomington, IN, USA). These tools offer a unique opportunity for the researcher

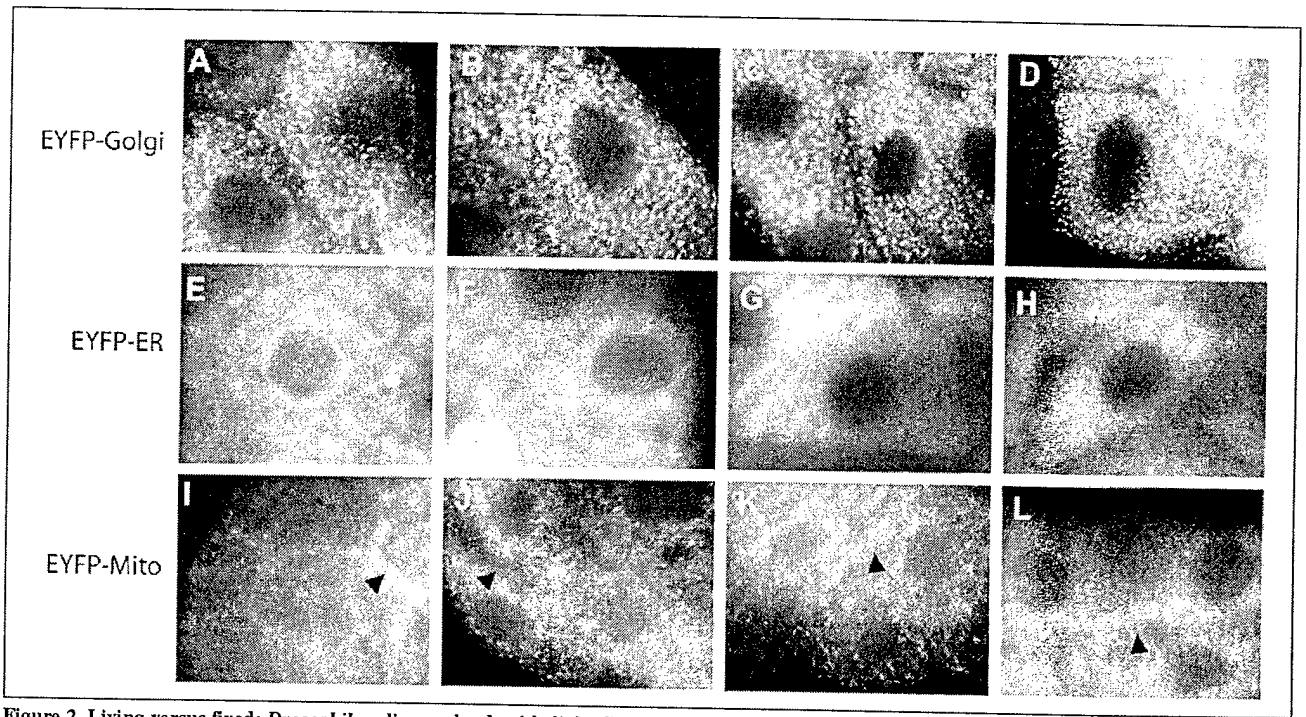

Figure 2. Living versus fixed: *Drosophila* salivary gland epithelial cells expressing enhanced yellow fluorescent protein (EYFP) membrane-targeted fixed under different buffer conditions. Living salivary gland epithelial cells expressing (A–D) EYFP-Golgi, (E–H) EYFP-ER, and (I–L) EYFP-Mito. (B, F, and J) Cells fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS). (C, G, and K) Cells fixed with 2% paraformaldehyde in PIPES/EGTA/magnesium (PEM) buffer. (D, H, and L) Cells fixed with 2% paraformaldehyde in phosphate lysine periodate (PLP) buffer. Fixation under a variety of buffer conditions results in little alteration to the overall distribution of EYFP-Golgi localization (compare A and B, C and D). However, we observed consistent reduction in the size of Golgi EYFP bodies when fixed in the PLP buffer. In living larval salivary gland epithelial cells, EYFP-ER localizes to a large cytoplasmic network (E), but fixation destroyed all discernable structure of the label (F, G, and H). EYFP-Mito localization remains generally unaltered after fixation, however, the cortical localization of EYFP-Mito is lost with fixation (I) to cells borders as defined by arrowheads in (J, K, and L).

studying intracellular organization and organelle structure. Although there are antibody markers and other GFP fusion proteins to endogenous endoplasmic reticular, mitochondrial, and Golgi-targeted proteins for use in *Drosophila* research (6,9–11), the new intracellular membrane markers reported in this report have distinct advantages over these tools. Since these constructs are merely EYFP moieties targeted to a specific organelle, they will therefore be less likely to affect the structure and organization of these organelles than ectopic overexpression of an endogenous protein. Moreover, the targeted EYFP constructs described in this paper are ubiquitously expressed and are not constructed in the bipartite UAS/GAL system (12); therefore, observation of the EYFP does not require the addition of further genetic elements, thus expediting mutant analysis and phenotypic characterization.

Using these constructs, we also addressed the topic of fixation artifact. An overwhelming majority of information regarding protein subcellular localization has been determined by common immunohistochemical techniques in which a tissue is fixed (usually with a formaldehyde-based fixative), incubated with a primary antibody specific to the target protein, and then labeled with secondary antibodies for detection (7). However, astonishingly little work has been published regarding the extent and role that fixation artifact plays in this process, although differences between living and fixed tissue have been alluded to in the literature and anecdotally. Traditionally, fixation artifact has been of greater concern in electron microscopy, and through the development of some advanced procedures such as freeze substitution, a few of these obstacles have been overcome (8). In most immunohistochemical procedures, there is a conflict between fixation and preservation of the epitope. Generally, longer and stronger fixations preserve more of the overall cellular and intracellular structure, but the cost is the loss of the sensitive epitopes that are recognized by the primary antibodies. As shown with immunoelectron microscopy, the fixation of organelles composed of membranes with chemical cross-linking agents such as paraformaldehyde presents unusual problems. Biological membranes are not entirely composed of proteins and therefore do not contain moieties that can be cross-linked, and it has been suggested that fixation with cross-linking agents may even alter apparent membrane structure through chemical cross-linking (8).

Here we show that fixation alters the morphology of intracellular organelle structure. However, the extent of artifact was dependent on a fixative as well as organelle. Some structures fixed well under a variety of conditions (Golgi and mitochondria), while the endoplasmic reticulum was extremely labile to any fixation protocol. In all procedures, we observed differences between fixed and living specimens. Some features such as the intracellular organization of EYFP-Golgi and the intracellular distribution of EYFP-Mito changed after fixation. However, the most striking results were the effects that a short wash with a buffered detergent solution had on our EYFP markers. Since most immunohistological techniques that are used to investigate intracellular antigens involve a permeabilization step to allow the antibody access to the target protein inside the cell, we examined the effects this procedure has on the localization of our membrane tags. In every case that a permeabilization/wash step was used after fixation with a 2% paraformaldehyde in any buffer formulation, all EYFP expression was lost. This artifact was solely dependent on paraformaldehyde concentration as the use of a 4% solution in any buffer resulted in the preservation of EYFP. The point of these experiments was not to define the conditions of fixation of a given organelle but to provide experimental caveats for future investigation and demonstrate the artifacts that such procedures generate. In general, we found that a 4% paraformaldehyde fixative in a PBS buffer preserved most of the structure and integrity of EYFP-Golgi and EYFP-Mito localization even after a PBT wash, while EYFP-ER fixed poorly under all conditions. However, these fixation conditions might not be appropriate for all epitopes found in an intracellular membrane compartment, and therefore it is appropriate to test a variety of different fixation conditions.

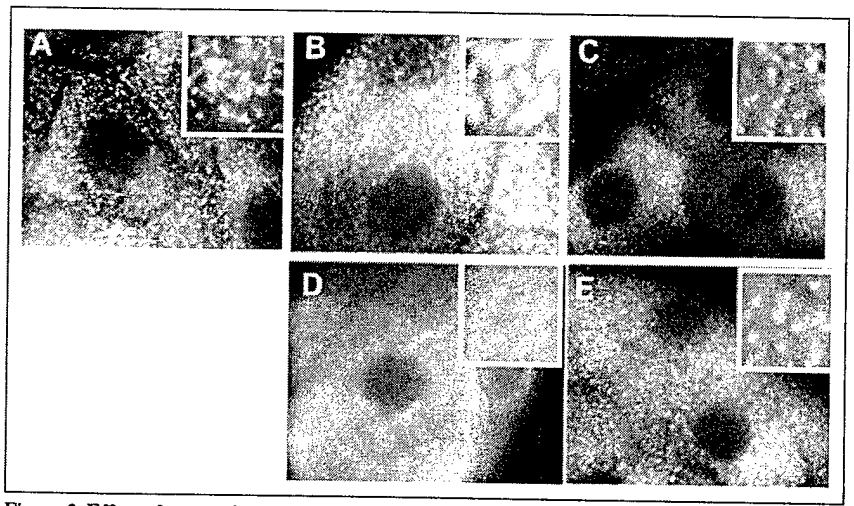

Figure 3. Effect of permeabilization on enhanced yellow fluorescent protein (EYFP)-Golgi in *Drosophila* larval salivary epithelial cells. Living larval salivary gland epithelial cells with (A) EYFP-Golgi; inset, the EYFP-Golgi structures are not round but are composed of multiple lobed structures. (B) Larval salivary gland epithelial cells fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) buffer; inset, amorphous, swollen appearance of Golgi bodies. (C) Larval salivary gland epithelial cells fixed with 4% paraformaldehyde in PBS buffer; inset, small, more discrete appearance of EYFP-labeled structures. (D) Cells fixed with 2% paraformaldehyde and then treated with 30 min incubation with 1× PBS, 1% bovine serum albumin (BSA), and 0.1% Triton X-100 (PBT); inset, no Golgi labeling. (E) Cells fixed with 4% paraformaldehyde in PBS buffer and then treated with 30 min incubation with PBT; inset, preservation of some Golgi staining.

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REFERENCES

1. **Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward, and D.C. Prasher.** 1994. Green fluorescent protein as a marker for gene expression. Science *263*:802-805.
2. **Rizzuto, R., M. Brini, P. Pizzo, M. Murgia, and T. Pozzan.** 1995. Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells Curr. Biol. *5*:635-642.
3. **LaJeunesse, D.R., B.M. McCartney, and R.G. Fehon.** 1998. Structural analysis of *Drosophila* merlin reveals functional domains important for growth control and subcellular localization. J. Cell Biol. *141*:1589-1599.
4. **McCartney, B.M. and R.G. Fehon.** 1996. Distinct cellular and subcellular patterns of expression imply distinct functions for the Drosophila homologues of moesin and the neurofibromatosis 2 tumor suppressor, merlin. J. Cell Biol. *133*:843-852.
5. **Wolff, T.** 2000. Histological techniques for the Drosophila eye part I: larva and pupa, p. 210-227. *In* W. Sullivan, M. Ashburner, and R.S. Hawley (Eds.), *Drosophila* Protocols. CSH Laboratory Press, New York.
6. **Bobinnec, Y., C. Marcaillou, X. Morin, and A. Debec.** 2002. Dynamics of the endoplasmic reticulum during early development of *Drosophila melanogaster* Cell Motil. Cytoskeleton *54*:217-225.
7. **Rebay, I. and R. Fehon.** 2000. Histological techniques for the Drosophila eye part I: larva and pupa, p. 389-411. *In* W. Sullivan, M. Ashburne, and R.S. Hawley (Eds.), *Drosophila* Protocols. CSH Laboratory Press, New York.
8. **Moreira, J.E., V. Dodane, and T.S. Reese.** 1998. Immunoelectronmicroscopy of soluble and membrane proteins with a sensitive postembedding method. J. Histochem. Cytochem. *46*:847-854.
9. **Jonaki, S., J.S. Goltz, M. Konsolaki, T. Schüpbach, and D. Stein.** 2000. Windbeutel is required for function and correct subcellular localization of the Drosophila patterning protein Pipe. Development *127*:5541-5550.
10. **Santel, A., N. Blumer, M. Kampfer, and R. Renkawitz-Pohl.** 1998 Flagellar mitochondrial association of the male-specific Don Juan protein in Drosophila spermatozoa. J. Cell Sci. *111*:3299-3309.
11. **Sisson, J.C., C. Field, R. Ventura, A. Royou, and W. Sullivan.** 2000. Lava Lamp, a novel peripheral golgi protein, is required for *Drosophila melanogaster* cellularization. J. Cell Biol. *151*:905-917.
12. **Brand, A.H. and N. Perrimon.** 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*:401-415.

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